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Two Types of cDNAs Encoding Proopiomelanocortin of Sockeye Salmon, *Oncorhynchus nerka*

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ABSTRACT—To investigate regulatory mechanisms of proopiomelanocortin (POMC) gene expression in sockeye salmon, we have isolated and characterized cDNAs encoding two types of sockeye salmon POMC, which are referred to as ssPOMC-A and -B. Two types of PCR products were amplified from total RNA of sockeye salmon pituitaries by use of rainbow trout sequences. Full length cDNA clones encoding ssPOMC-A and ssPOMC-B were obtained from a pituitary cDNA library of sockeye salmon using the PCR products as probes. The ssPOMC-A and -B cDNAs have a length of 1072 and 1709 bps, respectively. Northern blot analysis showed that both ssPOMC-A and -B mRNAs were expressed only in the pituitary, and their sizes were about 1.2 kb and 1.8 kb, respectively. The presence of two ssPOMC genes was confirmed by Southern blot analysis of genomic DNA obtained from a single sockeye salmon. The deduced amino acid sequences of the ssPOMC-A and -B contained 230 and 226 residues, respectively. The amino terminal of β-endorphin in ssPOMC-B which corresponds to Met-enkephalin domain is YSGFM, which is different from YGGFM of Met-enkephalin found in many other vertebrate species. The homology of nucleotide sequences between ssPOMC-A and -B is 59% in the entire coding region, whereas α-MSH coding regions are highly homologous (91%). Although the deduced amino acid sequences of ssPOMCs show 43% overall similarity, their hydropathy profiles are coincident with those of several other vertebrate species, particularly the amino terminal of N-terminal peptide (NPP) shows almost the same pattern with other vertebrate NPPs.

INTRODUCTION

Proopiomelanocortin (POMC) is the precursor for a number of biologically active peptides such as adrenocorticotropin (ACTH), α-melanophore-stimulating hormone (α-MSH) and β-endorphin. The complete amino acid sequences of POMC, which were deduced from the nucleotide sequences of cDNA, were reported in several mammals (Nakanishi *et al.*, 1979; Drouin and Goodman, 1980; Uhler and Herbert, 1982; Keightley *et al.*, 1991), amphibians (Martens *et al.*, 1985; Hilario *et al.*, 1990) and fishes (Kitahara *et al.*, 1988; Salbert *et al.*, 1992). It is well known, in mammals, that stressful stimuli increased the levels of plasma ACTH and pituitary POMC mRNA (Aguilera, 1994). In teleost fish, the plasma ACTH levels were similarly increased by stressful stimuli (Sumpter *et al.*, 1986; Balm and Pottinger, 1995), however, the level of POMC gene expression was not analyzed in the previous studies.

Juveniles of many salmonid species migrate to the ocean or the lake where they spend several years, and return their natal river to spawn. The juveniles must adapt to novel environmental circumstances. As reported in mammals, the adaptation to novel environments should be stressful for the fish. It is thus important to investigate the changes in expression of POMC gene and its regulatory mechanisms in salmonids.

Because of tetraproidy (Ohno *et al.*, 1968), salmonid fishes often have two types of genes for a certain hormone. The structures of two different cDNAs were determined for precursors of salmon gonadotropin-releasing hormones in sockeye salmon (Ashihara *et al.*, 1995), and for those of melanin-concentrating hormone in chum salmon (Ono *et al.*, 1988). The structures of two cDNAs were analyzed also for precursors of neurohypophysial hormones, vasotocin and isotocin, in chum salmon (Heierhorst *et al.*, 1990; Hyodo *et al.*, 1991; Suzuki *et al.*, 1992). The isolation and sequence analysis of chum salmon POMC-derived peptides suggested the presence of two POMC genes in salmon (Kawauchi, 1983). In fact, two different POMC cDNAs corresponding to POMC-A and -B, which are not highly homologous each other, were evidenced in rainbow trout (Salbert *et al.*, 1992).

In the present study, we have tried to isolate and

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characterize two different cDNAs for sockeye salmon POMCs, since it is important for understanding of neuroendocrine mechanisms of salmon migration, and also to initiate studies on the regulation of POMC gene expression in response to stressful stimuli during migratory behavior. Actually we could obtain two types of POMC cDNAs. Further, the result of Southern blot analysis demonstrated that genes which encode the two types of POMCs can be found in the same individual fish. Such information would be critical in the next steps of molecular study to analyze regulatory mechanisms of POMC gene expression in salmonids, as have been shown in two types of genes encoding salmon gonadotropin-releasing hormone precursors (Higa *et al.*, 1995).

MATERIALS AND METHODS

Fish

Immature (2+) sockeye salmon, *Oncorhynchus nerka*, of both sexes were obtained from the Toya Lake Station for Environmental Biology. Immediately after decapitation, brains, pituitaries, hearts, gills and kidneys were taken out and frozen in liquid nitrogen, and were stored at -80°C.

RNA and DNA preparation

Total RNA was prepared from 300 sockeye salmon pituitaries by means of guanidium thiocyanate/hot phenol method (Chirgwin *et al.*, 1979). For construction of cDNA library, poly(A)$^+$ RNA was purified from 1 mg of total pituitary RNA with Oligotex-dT30 (Japan Synthetic Rubber/Nippon Roche) according to the supplier's instruction. For Northern blot analyses, poly(A)$^+$ RNA was further prepared from the brains and single heart, gill and kidney of sockeye salmon.

For Southern blot analysis to confirm the approximate number of POMC genes, genomic DNA was extracted from the liver of single sockeye salmon.

Construction of cDNA library

Five micrograms of poly(A)$^+$ RNA from sockeye salmon pituitaries was used for double-strand cDNA synthesis. The double-strand cDNA was synthesized by the method of Gubler and Hoffman (1983) using a cDNA synthesis kit (Pharmacia). An *Eco*RI/*Not*I adapter was ligated to both 5' and 3' ends, and the cDNAs were fractionated by gel filtration on a Spun column (Pharmacia) and ligated into the *Eco*RI site of λZAPII vector (Stratagene). The resulting mixture was packaged into bacteriophage head using a commercial extract (Gigapack Gold, Stratagene). A library of approximately 3 x 10^5 cDNA clones was obtained.

RT-PCR

Oligonucleotide primers: The oligonucleotides for the PCR amplification were synthesized upon consideration of nucleotide sequences of rainbow trout and chum salmon POMC cDNAs (Salbert *et al.*, 1992; Kitahara *et al.*, 1988). The MSH primer (5'-TAC TCC ATG GAG CAC TCC CGC TGG-3') corresponds to the sequence for the α-MSH(2-9) in rainbow trout POMC-A, and the E1 primer (5'-CAT GAA GCC ACC GTA GCG CTT-3') to that for the β-endorphin(1-5) region with dibasic cleavage site also in rainbow trout POMC-A. These sequences are highly homologous among many vertebrate species hitherto examined. The E2 primer (5'-GGA TTG CTT GGT ATA TGG CTT CAT-3') follows the sequence corresponding to the β-endorphin (5-12) region in the chum salmon POMC which is homologous to that in rainbow trout POMC-B. This primer is specific to chum salmon POMC and rainbow trout POMC-B.

Polymerase chain reaction (PCR): The first-strand cDNA was synthesized from 5 μg of the total RNA of sockeye salmon pituitaries using a cDNA synthesis kit (LIFE SCIENCES, INC.). The PCR reaction mixture contained 1 μl of cDNA solution (1/25 vol.) described above, 5 μl of reaction buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$ and 0.01% (w/v) gelatin), 4 μl of dNTPs (2.5 mM), 2.5 μl of MSH primer (4 μM), 2.5 μl of E1 or E2 primer (4 μM), 1.25 units of *Taq* polymerase (Takara) and sterile distilled water to 50 μl, overlaid with 100 μl of mineral oil. The PCR was carried out in 30 cycles. Amplification conditions consisted of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min. The PCR product was electrophoresed on a 2.5% NuSive gel (FMC Co.) and purified by the phenol-chloroform extraction.

Sequence analysis of PCR products

The purified PCR products were phosphorylated in a mixture containing 50 mM Tris-HCl (pH 9.5), 10 mM $MgCl_2$, 5 mM DTT, 5% glycerol, 1 mM ATP and 10 units of T_4 polynucleotide kinase (Toyobo) at 37°C for 30 min and was precipitated with ethanol. Afterward, the phosphorylated fragments were ligated into SmaI-cut pBluescript II vector (Stratagene), and the nucleotide sequences of these clones were determined on both strands according to the dideoxy chain termination method (Sanger *et al.*, 1977) using a SQ-5500 DNA sequencer (Hitachi).

Isolation and sequence analysis of POMC-A and -B cDNA clones

The amplified library was screened using plaque hybridization with the PCR products as probes. The probes were labeled with a Megaprime DNA labeling system and [α-^{32}P]dCTP (Amersham). The E1 and E2 primers were used for labeling of probes. Hybridization was performed in a buffer containing 5 x SSPE (1 x SSPE: 180 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA), 5 x Denhart's solution, 0.5% SDS and 200 μg/ml denatured yeast tRNA at 65°C overnight. The membranes were washed twice in 2 x SSPE/0.1% SDS at 65°C for 1 hr and 0.1 x SSPE/0.1% SDS at 65°C for 30 min, and were exposed to Kodak X-ray film.

The inserts from positive cDNA clones were subcloned into pBluescript II plasmid (Stratagene) by the *in vivo* excision, and the nucleotide sequences of these clones were determined on both strands by sequencing of restricted partial sequences and deleted mutants of these clones. Nucleotide and amino acid sequences and polypeptide hydropathy profiles were analyzed using a genetic information processing program (GENETYX, Software Development Co, Ltd).

Northern and Southern blot analyses

The poly(A)$^+$ RNAs extracted from the brains, pituitaries, single heart, gill, liver, and kidney were analyzed by Northern blot method. They were electrophoresed in a 1% agarose/formaldehyde gel and transferred to Hybond-N+ membrane (Amarsham). For Southern blot analysis, 10 μg of DNA digested with a restriction enzyme, *Eco*RI, *Hin*dIII, or *Apa*I, was electrophoresed in a 0.8% agarose gel and transferred to Hybond-N+ membrane (Amarsham). Hybridization was carried out as described above. After washes of membranes, Northern blots were exposed to Kodak X-ray film overnight. Southern blots were exposed to Fuji imaging plate for 3 hr, and were analyzed by a bio-imaging analyzer system, Fujix Bas 2000 (Fuji Photo Film Co., Ltd).

RESULTS

Nucleotide sequences of sockeye salmon POMC cDNAs

Two types of PCR products encoding POMC were amplified from the total RNA of sockeye salmon pituitaries. The partial sequences of POMC-A and POMC-B corresponding to the rainbow trout POMC cDNAs (Salbert *et al.*, 1992) were obtained in the PCR products using E1 and

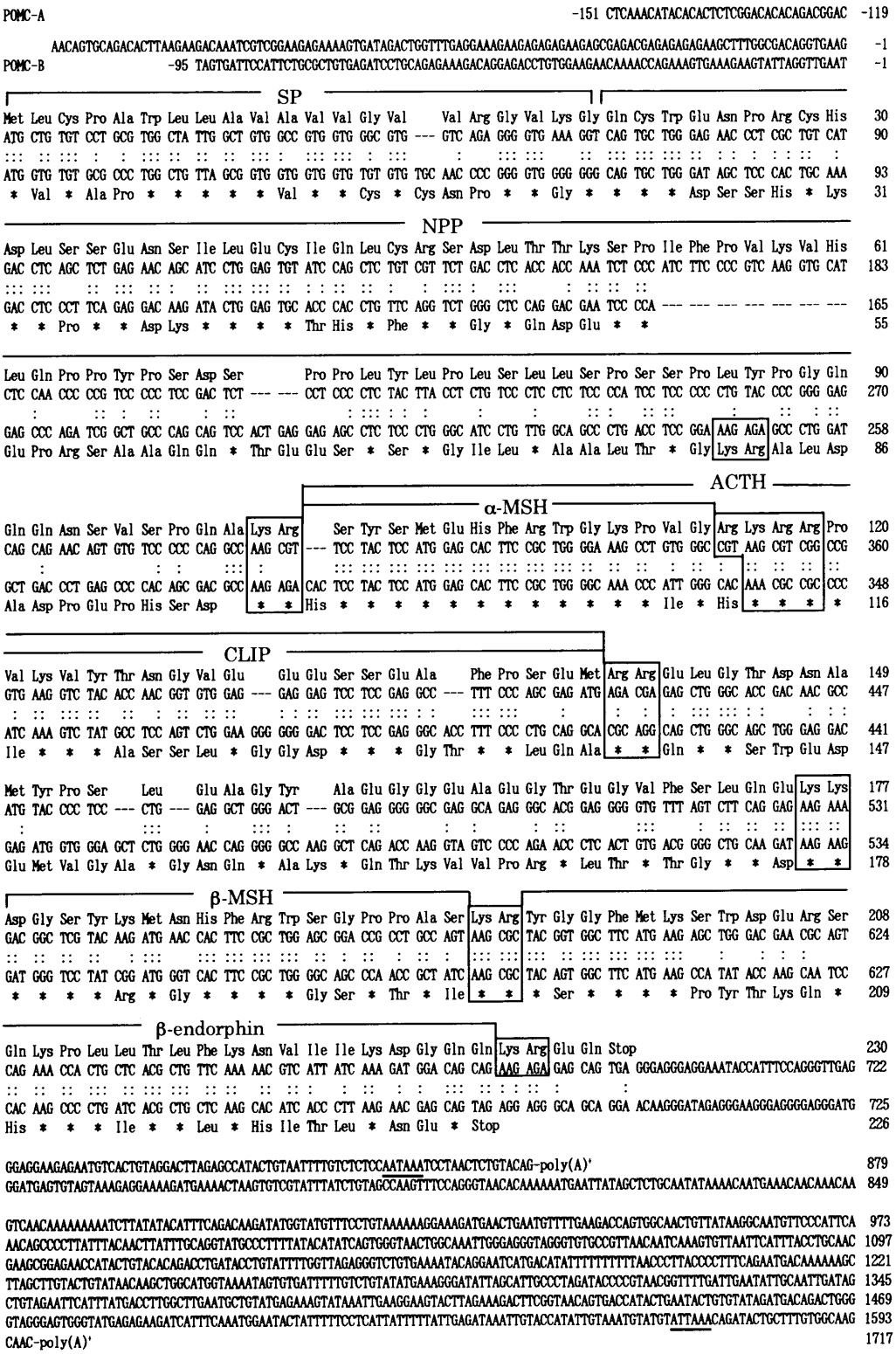

Fig. 1. Nucleotide sequences of cDNAs encoding sockeye salmon POMCs (POMC-A and -B) and deduced amino acid sequences. Nucleotides and the amino acid residues are numbered, beginning with the first residues in the coding region. Identical nucleotides and amino acids are indicated by colons and asterisks, respectively. Gaps are indicated by hyphens. Boxes show the potential dibasic cleavage sites. The underlined sequence in the 3'-nontranslated region indicates the polyadenylation recognition site. ACTH, adrenocorticotropin; CLIP, corticotropin-like intermediate lobe peptide; MSH, melanophore-stimulating hormone; NPP, N-terminal peptide; SP, signal peptide.

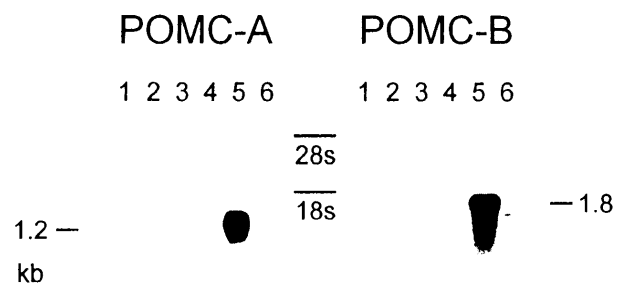

Fig. 2. Northern blot analysis for sockeye salmon POMC-A and -B mRNAs. Four micrograms of poly(A)$^+$ RNA from brains (lane 1), heart (lane 2), gill (lane 3), kidney (lane 4), pituitaries (lane 5) and liver (lane 6) were electrophoresed and hybridized with [α-^{32}P]-labeled sockeye salmon POMC-A and -B PCR products as described in the text. The positions of the 18S and 28S rRNAs are indicated between the blots.

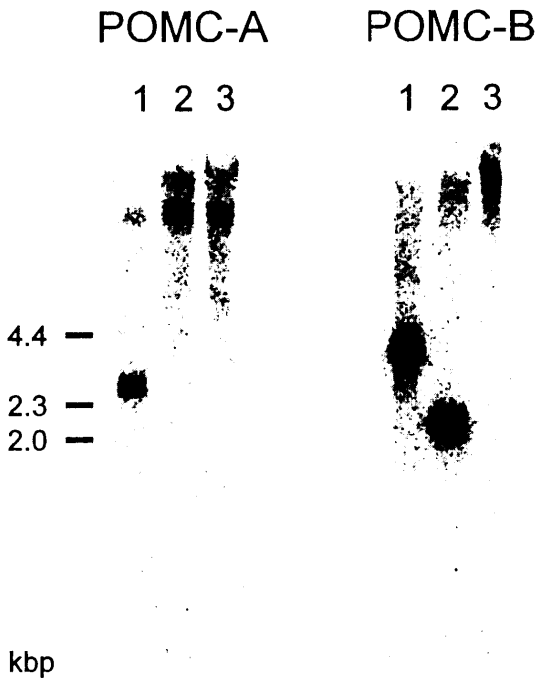

Fig. 3. Southern blot analysis of sockeye salmon POMC-A and -B genes in a single fish genome. Ten micrograms DNA from the liver was digested separately with restriction enzymes *Eco*RI (lane 1), *Hin*dIII (lane 2) or *Apa*I (lane 3), electrophoresed and hybridized as described in the text. Probes used for hybridization are shown above the lane numbers. Positions of size markers were obtained by use of *Hin*dIII and *Eco*RI double-digests of lambda DNA.

E2 primers, respectively. By screening of approximately 5 x 10^5 transformants, 1 for POMC-A and 2 for POMC-B positive clones were obtained using PCR products as probes. The sockeye salmon (ss) POMC-A cDNA has a length of 1072 bps and the deduced amino acid sequence is composed of 230 residues, whereas the ssPOMC-B has a length of 1709 bps and the deduced amino acid sequence contains 226 residues (Fig. 1).

The homology of nucleotide sequences between cDNAs encoding ssPOMC-A and -B is 59% in the entire coding region. The overall homology of the deduced amino acid sequences between ssPOMC-A and -B is 43%. However, hormone coding regions, particularly α-MSH coding region, which shows 91% homology, are well conserved not only in salmonids, but also in all classes of vertebrates. The same types of POMCs are highly homologous between sockeye salmon and rainbow trout (89% in POMC-A and 92% in POMC-B).

Northern and Southern blot analyses

Tissue-specific expression of POMC-A and -B genes was investigated by the Northern blot analysis (Fig. 2). The POMC-A and -B probes hybridized only with poly(A)$^+$ RNA obtained from the pituitaries. The lengths of POMC-A and -B mRNAs were approximately 1.2 kb and 1.8 kb, respectively.

Genomic DNA obtained from a single sockeye salmon was digested with *Eco*RI, *Hin*dIII, or *Apa*I and was hybridized with [α-^{32}P] labeled POMC-A and -B PCR products as probes. The difference in electrophoresed patterns among hybridized fragments indicates that an individual salmon probably has single copies of genes encoding each of POMC-A and -B (Fig. 3).

Comparison of the deduced amino acid sequences among ssPOMCs and other vertebrate POMCs.

As was reported on the rainbow trout and the chum salmon POMCs, the lack of γ-MSH sequence in ssPOMCs was confirmed by alignment of the deduced amino acid sequences with other vertebrate POMCs (Fig. 4). Well conserved regions were found in the amino terminal of NPP, ACTH, β-MSH and β-endorphin domains. Particularly, α-MSH is highly conserved over several vertebrate classes. ssPOMC-A is more similar to other vertebrate POMCs than POMC-B. ssPOMC-B contains a potential dibasic cleavage site in the NPP domain. The amino terminal portion of β-endorphin corresponding to Met-enkephalin is YSGFM in ssPOMC-B. This sequence is different from typical Met-enkephalin, YGGFM, found in many other vertebrates.

The hydropathy profiles of the N-terminal peptide (NPP) of ssPOMCs were compared with those of rainbow trout, bovine and *Xenopus* POMCs (Fig. 5). In spite of low homology of their deduced amino acid sequences, the overall hydropathy profiles showed similar patterns (data not shown). Interestingly, the hydropathy profiles of the amino terminal of NPP showed almost the same pattern among these POMCs. Six amino acid residues in the amino terminal of NPP, Cys^8, Cys^{20}, Asp^{10}, Leu^{11}, Glu^{14}, Leu^{18} are considered to be necessary for processing and sorting of POMC molecules (Cool *et al.*, 1995). These amino acid residues were also conserved in the same positions in the NPP of sockeye salmon POMCs.

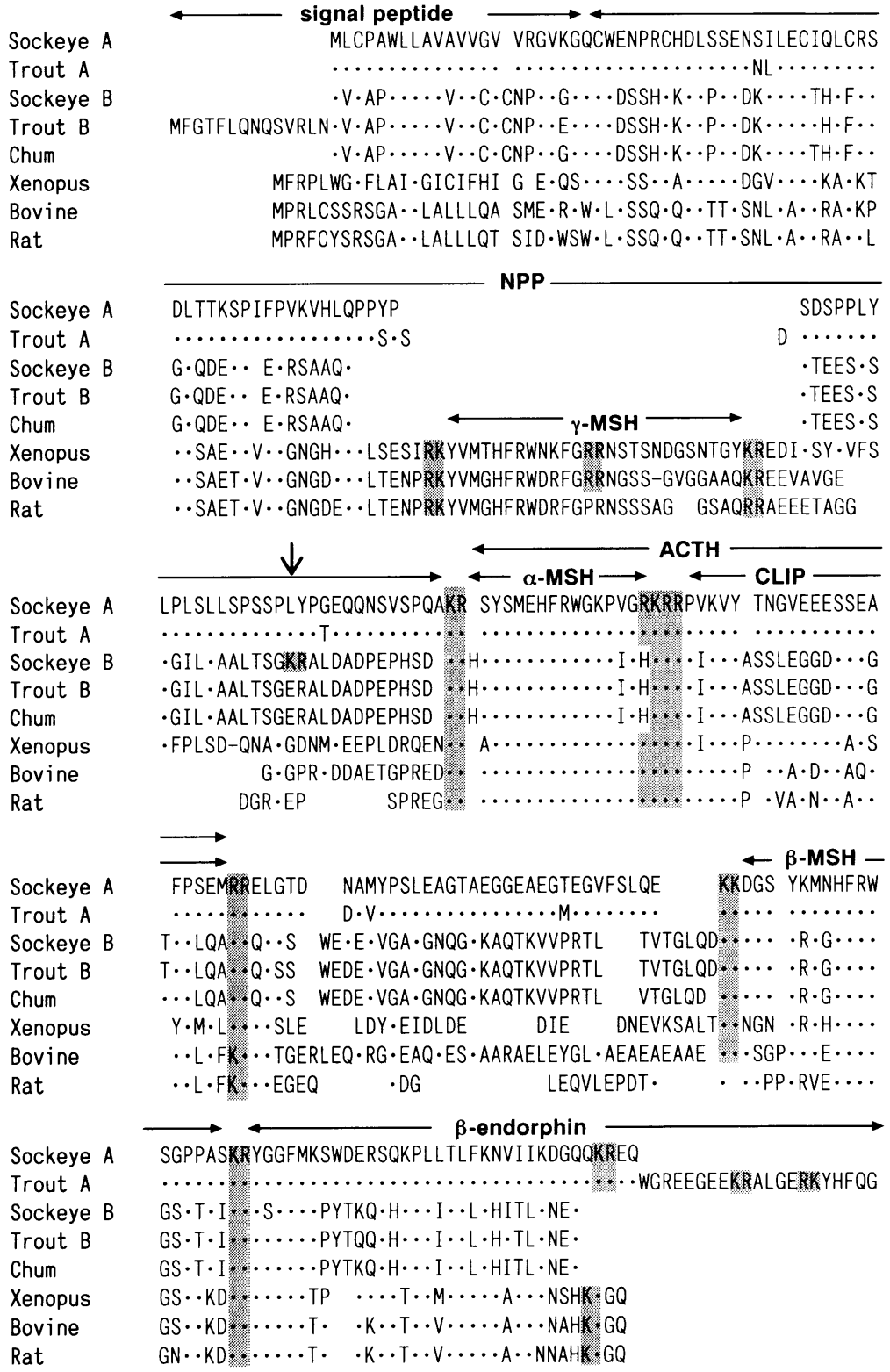

Fig. 4. Alignment of the amino acids sequences of POMCs from sockeye salmon, rainbow trout, chum salmon, *Xenopus*, bovine and rat. Dots indicate residues identical to that of sockeye salmon POMC-A sequence. Gaps have been introduced to maximize homology. The potential dibasic cleavage sites are shaded. An arrow indicates the sockeye salmon POMC-B specific cleavage site. All sequences are deduced from cDNA sequences which are taken from Salberts *et al.* (1992) for trout, Kitahara *et al.* (1988) for chum salmon, Martens *et al.* (1985) for *Xenopus*, Nakanishi *et al.* (1979) for bovine, and Drouin and Goodman (1980) for rat.

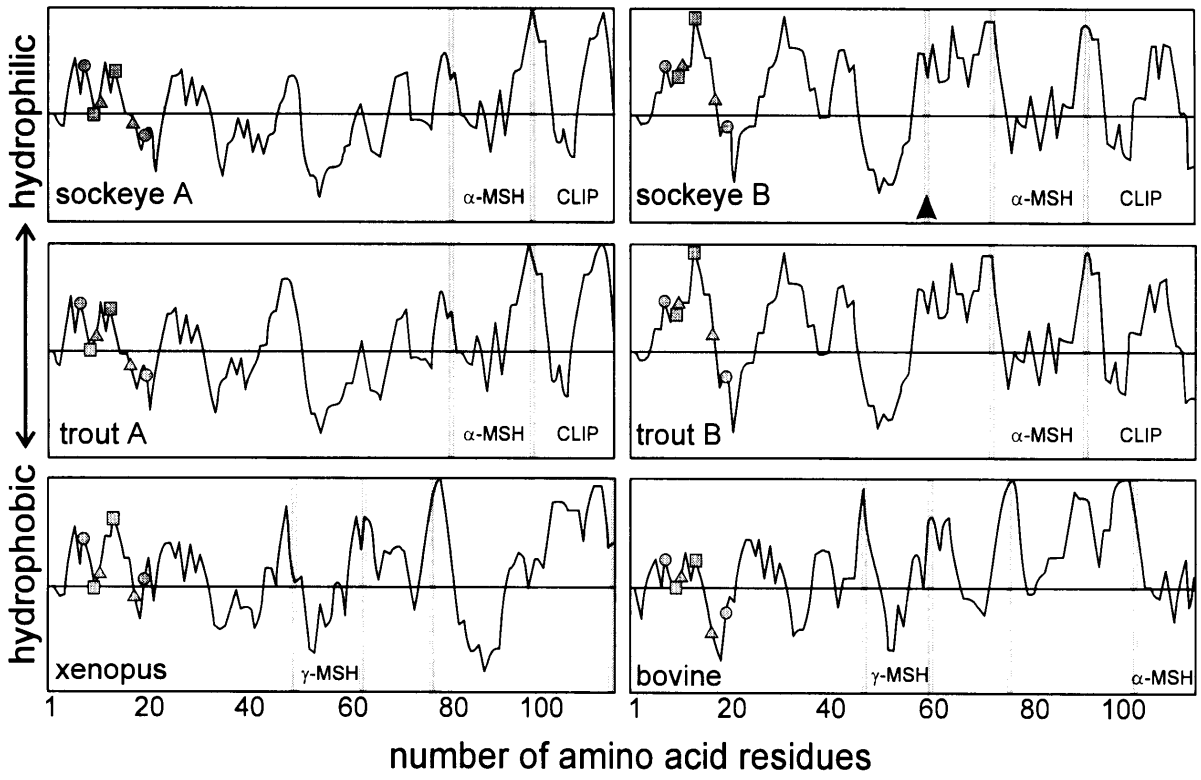

Fig. 5. Hydropathy profiles for the amino terminal region of POMC of sockeye salmon, rainbow trout, *Xenopus* and bovine according to Hopp and Woods (1981). All profiles were drawn from deduced amino acid sequences described in Fig. 4. Numbers below the figures indicate the positions of amino acid residues referring the first residue of NPP as 1. The potential dibasic cleavage sites are shaded. A solid triangle indicates the sockeye salmon POMC-B specific cleavage site. Circles, cysteine residues (Cys^{8} and Cys^{20}); squares, acidic amino acid residues (Asp^{10} and Glu^{14}); triangles, hydrophobic amino acid residues (Leu^{11} and Leu^{18}). Note that the positions of these amino acid residues are coincident in the amino terminal of NPPs.

DISCUSSION

Two types of POMC cDNAs were obtained from the pituitary of sockeye salmon, and the deduced precursors were referred to as POMC-A and -B following the previous report on rainbow trout (Salbert *et al.*, 1992). Southern and Northern blot analyses confirmed the presence and expression of two different POMC genes in the sockeye salmon. As has been discussed in the introduction, salmonid fishes frequently have two genes for a certain hormone, probably because of tetraploidization which is considered to occur about 100 million years ago in salmonid (see Urano *et al.*, 1992). The isolation and sequence analysis of POMC-derived peptides suggested the presence of two POMC genes in chum salmon (Kawauchi, 1983). However, only POMC-B cDNA was obtained from chum salmon pituitaries (Kitahara *et al.*, 1988). This discrepancy may be accounted for by the fact that the oligonucleotide probe used for screening of POMC cDNA in chum salmon was unique to a portion of POMC-B-derived β-endorphin. Since the sequence of this probe has low homology with the corresponding portion of POMC-A cDNA, Kitahara *et al.* (1988) might fail to detect POMC-A cDNA in chum salmon. Recently, the presence of two types of POMC mRNAs has been shown by Northern blot analysis of chum salmon pituitary mRNA which has actually detected two mRNAs, one for POMC-A and the other for POMC-B (Hiraoka *et al.*, 1995). We now convince that salmonid fishes have two types of POMC genes, as have been reported for other hormones (see Urano *et al.*, 1994), although the levels of expression of two genes remained to be determined.

Comparison of the nucleotide sequences between the ssPOMC-A and -B cDNAs showed that the hormone coding regions are highly conserved, whereas other regions, such as 5' and 3' non-coding regions and the internal sequence between ACTH and β-MSH coding regions, are considerably diverged. This coincidence in the structure of cDNAs suggests that two types of salmon POMC genes were derived from a single ancestral POMC gene by duplication. Since overall nucleotide homology between POMC-A and -B cDNAs is 59%, which is similar to those for salmon vasotocin and isotocin cDNAs (Suzuki *et al.*, 1992), we assume that the divergence of ancestral POMC gene occurred at the occasion of salmonid tetraploidization, as well as vasotocin and isotocin genes.

β-endorphins have the common amino acid sequence to other opioid peptides, YGGFM (or L), in first five amino acid residues and this sequence shows the activity as endogenous opiates (Goldstein, 1976). This sequence is highly conserved in almost all vertebrates. The lamprey, which is a member of

the oldest extant class of vertebrates, has proopiomelanotropin (POM) and proopiocortin (POC), whose amino acid sequences were recently deduced from the cDNA sequences (Takahashi *et al.*, 1995). The amino acid sequences of POM and POC showed that the YGGFM sequence was also conserved in the jawless fish β-endorphins. However, the β-endorphin domain of ssPOMC-B cDNA includes the different sequence, YSGFM, suggesting that the β-endorphin homologue derived from ssPOMC-B no longer has any opioid activity, but has different physiological function.

The first 26 amino acid residues of NPP, which contain 4 cysteine residues, are highly conserved in all vertebrate classes, and have been experimentally shown to form a hairpin loop which is stabilized by two disulfide bridges. Particularly, the NPP Cys^{8} to Cys^{20} domain and 4 hydrophobic and acidic amino acid residues (Asp^{10}-Leu^{11}-Glu^{14}-Leu^{18}) in this structure are necessary for sorting and transport of POMC molecules through a regulated secretory pathway (Cool *et al.*, 1995). In the sockeye salmon POMCs, these 6 amino acid residues are well conserved in NPP. The same amphipathic pattern in this region was further shown by comparison of the hydropathy profiles of POMCs in several other vertebrates. These results indicate that similar processing and sorting mechanisms of POMCs are highly conserved in vertebrates, and also suggest that secretion of both POMC-A and -B are similarly regulated in the salmon pituitary.

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